Specification

Anti NC1 Monoclonal Antibody

Technical field

The present invention relates to a method and reagent of detecting nephritis using anti NC1 monoclonal antibody. Furthermore, therapeutic implements and medicines are embraced therein.

Background art

Main conventional parameters to detect or diagnose nephritis using the urine sample include the presence and/or level of a protein, albumin, type IV collagen (three chains-domain) and β2M etc. Further, conventional definite diagnostic method for nephritis depends on a method of staining renal slice samples obtained by renal biopsy in order to observe the deposit of immunoglobulin(hereafter referred to as Ig) or formation of crescents. For example, in order to diagnose IgA nephropathy, detecting "granular deposit of IgA mainly in diffused mesangial region of kidney" may need to be done, for example, as described in p.1071 of Laboratory Examination (2001~ 2002; published by BUNKO-DO). The foregoing detecting granular deposit of IgA can be done by an immunofluorescence assay or enzyme immunoassay, however, it may necessarily require the renal biopsy procedures.

Disclosure of invention

Problems to be solved by the invention

These foregoing methods, however, include the following problems:

The definite diagnosis may need high diagnostic techniques of well experienced pathologists. In addition, at the time when the deposit of Igs is detected, the stage of the nephritis might have already progressed for a long period, sometime for several decades and its renal function might have remarkably decreased. Therefore, in order to diagnose nephritis more simply and more precisely, a better diagnostic method that can diagnose a kidney disease at a very early stage even before deposit of

Ig and/or formation of glomerular crescent can be observed has been aspired

Means for settlement of the problems

The present invention can diminish some problems as mentioned above and dedicates detection methods and detecting reagents for diagnosing nephritis in its early stage and further dedicates a sero-cleaning method.

The present inventors, heretofore, have suggested that a common antibody detected from various types of nephritis is specific to NC1 domain of type IV collagen. In fact, the present inventors have detected anti NC1 antibody with high frequency from the serum or urine in various types of nephritis including anti glomerular basement membrane(GBM) antibody nephritis. It has been reported that the antigen of the antibodies detected from the anti GBM antibody nephritis is a part of NC1 domain. There is no antibody prepared artificially that can bind the antigen only at the crisis. Furthermore, existence of a common antibody in various types of nephritis cannot have been detected by an immunohistofluorescent staining method. Thereupon, the present inventors prepared an anti NC1 monoclonal antibody by sensitizing the antigen NC1 isolated and purified from bovine renal glomeruli with the mouse and accomplished "Anti NC1 monoclonal antibody" and " Labeled anti NC1 monoclonal antibody". In some embodiments, such antibodies can be applicable to the Western blot method or immunofluorescent staining method, and further to a "NC1 detecting ELISA kit with sandwich technique".

The procedures are as follows:

1. **[Isolation and purification of antigen]** Isolate type IV collagen NC1 domain (hereinafter referred as NC1) from the raw material of bovine renal glomeruli and purify it by column chromatography (J. Biol. Chem., 263, 10481-8).

2. **[Preparation and selection of anti NC1 monoclonal antibody]** Prepare monoclonal antibody using the mouse by the usual method (Monoclonal Antibody Experimental Manual., published by KODAN-Sha, 1987).

For screening of the cell fusion, select a lot of positive pores showing high antibody titer from an ELISA assay and further select the antibody responding to a NC1 monomer and/or NC1 dimmer by Western blot techniques. Then, perform an

immunofluorescent staining and firstly select an antibody that can stain the sample from the monkey anti glomerular basement membrane (GBM) antibody nephritis but not the sample from the normal monkey kidney. The resulting anti NC1 monoclonal antibody can be applicable to not only ELISA method, Western blot method but also immunofluorescent stain method.

Of course, such anti NC1 monoclonal antibody is preferred to be applied to only one method or two or more methods among ELISA, Western blot, immunofluorescent stain or other methods. Such anti NC1 monoclonal antibody, however, is most preferable to be applied to any method. In some immunofluorescent stain methods, antibodies responding to a normal kidney and polyclonal antibodies prepared from the rabbit etc. are acceptable and useful to detect existence of the tissues. However, those antibodies may not be used to discriminate between nephritis and normal kidney.

A crab-eating macaque nephritis model can be established by dorsal injection of 1mg of initial dose and 3mg of booster dose of NC1 which is different from the previous reports using plantar injection. The merits of dorsal injection include no difficulties in walking and less infection in the animal. More specifically, in the dorsal injection, it is preferable to inject the larger booster dose compared to the initial dose rather than 4mg of single dose or smaller or equivalent booster dose compared to the initial dose. Further specifically, more than 1.5-fold booster dose is more preferable and 3-fold is most preferable compared to the initial dose.

This theory is usefully applicable to prepare type II collagen arthritis model etc. in sensitized animal or to provide various vaccinations. For example, in the case of vaccination of hepatitis type B, the conventional booster dose, sometime, may not increase its antibody titer. In such case, more than 1.5-fold booster dose higher than the conventional single dose or 1/2 initial dose is preferred. Also3-fold higher in booster dose than the conventional initial dose is more preferable.

“Anti NC1 monoclonal antibody” of the present invention stains the renal glomerular basement membrane(GBM) of the pathological sections obtained from monkey GBM antibody nephritis or human IgA nephropathy using an indirect

immunofluorescent stain method. Furthermore, this antibody stains similarly other pathological sections obtained from other animal species such as the rat and mouse etc. or other various kinds of human nephritis other than the IgA nephropathy. "NC1 assay ELISA kit" of the present invention is useful for the early detection of primary nephritis and secondary nephritis such as diabetic nephritis etc. More specifically, in " Anti GBM antibody nephritis", the anti NC1 monoclonal antibody reacts significantly with NC1 which is abundant in GBM at injury crisis. Therefore, it can obtain particularly good hypersensitivity and provide sensitive detection of NC1 in urine as well as in serum samples.

Furthermore, anti NC1 antibody in serum or urine of a HIGA mouse IgA nephropathy model can be detected by an ELISA method with an "Anti NC1 monoclonal antibody" as the positive standard. In any case of glomerular nephritis such as some types of nephritis, diabetes, hypertension models etc., an assay or detection of anti NC1 antibody may be a significant indicator of the disease progression. In the other cases of nephritis occurred from infection or other disease models, an assay or detection of anti NC1 antibody may also be a good indicator of the disease progression.

The present inventors established the following operative means for "NC1 assay ELISA kit" in order to detect nephritis in an early stage. More specifically, the detecting method and the assay reagents in order to detect NC1 using the urine sample obtained from anti GBM antibody nephritis patient as well as using the serum sample will be exemplified and detailed below. However, the present invention should not be construed as being limited thereto.

1. NC1 detecting method and assay reagents in serum and/or urine.

NC1 can be detected using the following reagents: 1) an Anti NC1 antibody(derived from rabbit)-coated plate, 2) an Enzyme(HRP)-labeled anti NC1 monoclonal antibody, 3) a Coloring substrate(TMB), 4) a Reaction stopper solution(sulfuric acid).

Hereupon, it is preferable to prepare non-labeled "2)" and add "Enzyme(HRP) labeled anti mouse IgG antibody" as "2)-2". Further, it is preferable to exchange their

antibody parts between "1)" and "2)" or to change the both into monoclonal antibody.

Hereunto, the positive standard is preferably obtained from the human patient but more preferably obtained from the monkey of an experimental model invented by the present inventors. It is because the more stable standard can be obtained from the monkey which is more carefully bred and well-controlled. More specifically, it is preferable to make the human patient sample to a primary standard and the sample derived from the monkey to a secondary standard that can be equipped in the assay kit.

Immune reaction assay methods include not only an enzyme immunoassay method which is representatively used but also an AB method, RIA method, immunoluminescence method, precipitation method, agglutination method etc. An enzyme labeled antibody in the enzyme immunoassay includes the both antibodies without distinction as to a polyclonal or monoclonal antibody. Furthermore, the antibody can be preferably equipped into a radio-labeled compound(RIA method), lumino-labeled compound(immunofluorescence method) or non-labeled compound(precipitation method, agglutination method).

The reaction mode includes not only a sandwich method but also a competitive method etc. The sandwich method is, however, specifically more preferable. For a composition of assay reagents, an anti NC1 antibody coated plate can preferably be made of a glass or magnetic substance, or a method without solid phase method that does not use plate is also preferably accepted.

When anti NC1 antibody(hereinafter referred to as the antibody) is coated on a plate, such a coating substance can preferably be indirectly coated on a plate and the coating substance may include adipic acid, biotin, or combined substance of them.

An antigen can preferably be prepared from bioextracts, recombinants, or constructive peptides (including specified fractions or synthesized materials). Then the antibody can preferably be prepared from these antigens.

The animal species of antigens using for the assay reagents include preferably not only a human but also a monkey, bovine, swine, chicken, sheep, goat, rabbit, rat etc, but not limited to them. Further, the antigen can preferably include mixed antigens

from multi-animal species.

The antigen-derived organ includes preferably the kidney, but not limited to it.

2. Anti NC1 antibody remover and/or NC1 remover.

NC1 in sera can only be removed by dialysis of the blood through an affinity column prepared with the anti NC1 antibody. The anti NC1 antibody in sera can only be removed by dialysis of the blood through an affinity column prepared with NC1. After these treatments, the blood from which both antibody and antigen are removed will be recycled into the internal circulation of the body. Application of this principle to the crab-eating macaque nephritis model {sensitized by [K35 NC1](provided by Collagen Research Center)} showed that the concentrations of both antigen and antibody in urine become less than half after treatment. In conventional dialysis, however, the concentrations of both the antigen and antibody in the patient serum may not show such differences after treatments as mentioned above. Of course, any remover of NC1 and/or anti NC1 antibody in serum may preferably be included, but not limited to the above mentioned removers. Further, it is preferable to replace NC1 or anti NC1 antibody with α 3-chain group, α 4-chain group and/or their antibodies. It is also preferable to replace them with α 3-chain antigen part, α 4-chain antigen part inducing anti GBM antibody nephritis and/or their antibodies.

Antibodies used in the remover include preferably either a polyclonal or monoclonal antibody and a monoclonal antibody is more preferable because it can be processed with semi-permanently constant activity in its property.

The removers of the present invention are specifically useful for some nephritis in need of urgent care such as as anti GBM antibody nephritis etc.

Effect of the invention

The present invention is useful for early detection or definite diagnosis of nephritis and for treatment of a nephritis patient or cancer patient.

Example 1

[Isolation and purification of antigen] Isolate NC1 domain of type IV collagen (hereafter referred to as NC1) from bovine renal glomeruli as the raw material and purify it using column chromatography (J. Biol. Chem., 263, 10481-8).

[Preparation and selection of antibody] Prepare antibodies using the mouse (Monoclonal Antibody Experimental Manual. KODAN-Sha. 1987). For screening of cell fusion, select a lot of positive pores which possess high antibody titer using an ELISA method. After cellular proliferation of them in the mouse abdominal cavity, collect the ascitic liquid and select the antibody which reacts with both NC1 monomer and NC1 dimmer (Fig.1). Then, perform an immunofluorescent staining using crab-eating macaque normal kidney and nephritis model kidney (anti GBM antibody nephritis) and select the antibody which reacts with the glomeruli of the crab-eating macaque anti GBM antibody nephritis but not with the crab-eating macaque normal kidney(Table 1, Fig. 2).

(The crab-eating macaque nephritis model of the present invention prepared hereupon, is different one in its administration site from the reported administration site previously. In this model of the present invention, the administration consists of 1 mg NC1 of the primary dorsal injection and 3 mg NC1 of the booster dorsal injection. Dorsal injection may not produce any difficulty in walking or in bipedal walking of the monkey and cause less infection compared to a plantar injection.)

[Experiment on NC1 injection in crab-eating macaque (female, 3 y.o.), 2 animals in each group]

1) Administration site and method; The same doses of both NC1 and FCA were injected intracutaneously on the dorsal site of the animal.

2) Assay of anti NC1 antibody titer in the urine (diluted into 50-fold)(Assays were done before administration and 4 weeks after initial administration)

Changes of titer mean values of 2 animals

- Single administration (4 mg) 0.018 ⇒ 0.087
- Twice administrations (3 mg initial, 1 mg booster after 3 weeks) 0.029 ⇒ 0.256
- Twice administrations (1 mg initial, 3 mg booster after 3 weeks) 0.006 ⇒ 1.037

3) Assay method; Urinal specimens were added onto a 96 wells micro-plate applied with NC1(5 μg/ml) derived from bovine and incubated for 2 hours at room

temperature. After washing, HRP labeled anti human IgG antibody was added and incubated for 1 hour at room temperature. After washing, a luminary substrate solution was initially added and after 10 minutes, a reaction stopper solution was secondarily added. Then, the absorbance at 450 nm of wave length (A450 nm) was immediately measured.

Furthermore, an anti human type IV collagen (antigen derived from placenta, pepsin treated) polyclonal antibody(derived from rabbit) and an anti NC1 polyclonal antibody(derived from rabbit) were prepared and their stainability was compared between in a monkey normal kidney and in a monkey nephritis model kidney using an indirect immunofluorescence method. While both the above kidneys were stained (Fig.3-1, Fig.3-2), the anti NC1 monoclonal antibody of the present invention is more stainable in the nephritis kidney rather than the in normal kidney.

Therefore, the anti NC1 monoclonal antibody of the present invention dedicates an useful staining reagent in order to detect nephritis.

In fact, the anti NC1 monoclonal antibody selected from the present invention can stain the kidney of human glomerulonephritis. For instance, the antibody can stain GBM and uriniferous tubule of the frozen kidney section from IgA nephropathy.

The antibody of the present invention can stain kidneys in various types of human nephritis not only in IgA nephropathy but also in renal GBM. The antibody can more specifically stain glomeruli, uriniferous tubule, Bowman's capsule etc. in a minimal-change type primary nephrosis or a diabetic nephropathy. However, it does not stain kidneys which include those of a recovered minimal-change type nephrosis or normal kidney (Fig.4). Furthermore, in order to confirm the specificity of this antibody, when its immunological response was examined by the Western blot method using type IV collagen (derived from human placenta, pepsin treated) and NC1(derived from bovine renal glomeruli, collagenase treated) as antigens, the antibody responded to NC1 but did not respond to type IV collagen(Fig. 5). The anti NC1 monoclonal antibody of the present invention can be applicable either to an ELISA method, Western blot method or to immnofluorescent stain method.

Example 2

[Anti NC1 antibody remover and/or NC1 remover]

NC1 in sera can only be removed by dialysis of the blood through an affinity column prepared with an anti NC1 antibody. The anti NC1 antibody in sera can only be removed by dialysis of the blood through an affinity column prepared with NC1. After these treatments, the blood from which both antibody and antigen are removed will be recycled into the internal circulation of the body. In accordance with this principle, the blood equivalent to 4 ml of serum was collected from a nephritis model of crab-eating macaque (female, approx. 3 y.o.) and dialyzed through two kinds of affinity columns described above and then, recycled into the internal blood circulation of the animal and this procedure was subsequently repeated 3 times. When the both antibody titers in urine before and after treatment were assayed, the antibody titer after treatment decreased into less than half compared to that before treatment.

Example 3

[Type IV collagen assay kit prepared with anti type IV collagen antibody made from type IV collagen antigen derived from kidney]

It is well known that type IV collagen has chains from α 1 to α 6. Constitution of α-chains may differ upon the kinds of sourcing organ. A Type IV collagen derived from placenta possesses mainly α 1- and α 2-chains. On the contrary, a type IV collagen of kidney origin possesses α 3- and α 4-chains abundantly compared to the placenta origin. In order to obtain the type IV collagen derived from a kidney of the present invention, the following procedures can be done:

extract the collagen from bovine renal GBM using an usual pepsin-degradation method; and

then, remove NC1 fine particles, which may be contaminated in this process, using anti NC1 affinity column prepared separately.

Consequently, pure type IV collagen derived from a kidney can be obtained through this process. At the same time, the antibody with specifically high titer can be obtained by making the type IV collagen to an antigen.

Measurement of antibody titer; The following procedures can be done

Coat the antigen with 1)Bovine renal glomeruli pepsin soluble type IV collagen and 2)human placental pepsin soluble type IV collagen onto a 96 wells plate;

add 100 μl of specimen;

after incubation for 2 hours at room temperature, add HRP labeled antibody

incubate them for one hour at room temperature;

add TMB solution and incubate them for 10 minutes at room temperature;

stop the reaction by 1N sulfuric acid stopper solution; and

measure immediately the absorbance at 450nm of wave length.

Specimens and assay results;

- Specimen/Anti human placental type IV collagen monoclonal antibody (Immune animal/mouse, 3 kinds; 1A, 1B, 1E) ;
  1) All minus(background deleted, the same bellow)
  2) 1A/1.826, 1B/2.188, 1E/2.222
- Specimen/Anti human placental type IV collagen polyclonal antibody (Immune animal/rabbit, YOKO203) ;
  1) 2.391
  2) 2.231
- Specimen/Marketed anti human placental type IV monoclonal antibody (Immune animal/mouse, F59)
  1) 0.047
  2) 2.135
- Specimen/Marketed anti human placental type IV polyclonal antibody (Immune animal/goat, GOAT) ;
  1) 0.450
  2) 2.037
- Only antibody YOKO responded to both bovine renal GBM pepsin soluble type IV collage and human placental pepsin soluble type IV collagen but other antibodies responded to only one of these type IV collagens.

Conclusion;

For the measurement of renal type IV collagen, the reagent prepared with the

antibody against renal type IV collagen antigen (ELISA kit etc.) is preferable.

Further, the renal type IV collagen antigen is preferable to use in assaying anti type IV collagen antibody for evaluation of renal function.

Example 4

[Assay of anti NC1 antibody in IgA nephropathy model HIGA mouse]

3 HIGA mice (female, 4 week aged) were purchased and bred for measurement. Test specimens were obtained from one sampling for blood specimen and several samplings per day for urine totalized specimen. The serum sample was diluted into 200-fold and the urine sample into 4-fold for measurement. An ELISA method was used for assay.

- Both IgA antibody and IgG antibody were detected in the sera of all animals aged 6 weeks or more.
- IgA antibody was detected in the urine of the animals aged 15 weeks and IgG antibody was detected in the urine of the animals aged 18 weeks.

Brief description of the drawings

Fig.1. Selection of antibody by Western blot method: Lane 17: Control; Poly.: Anti NC1 polyclonal antibody.

Fig.2. Comparison of staining with anti NC1 monoclonal antibody between crab-eating macaque normal kidney and its nephritis model kidney (Indirect immunohistofluorescent stain).

Fig.3-1. Comparison of staining with anti human type IV collagen (derived from placenta) polyclonal antibody (derived rabbit) between crab-eating macaque normal kidney and its nephritis model kidney (Indirect immunohistofluorescent stain).

Fig.3-2. Comparison of staining with anti NC1 polyclonal antibody(derived from rabbit) between crab-eating macaque normal kidney and its nephritis model kidney (Indirect immunohistofluorescent stain).

Fig.4. Staining of kidneys of various human nephritis with anti NC1 monoclonal antibody (Indirect immunohistofluorescent stain).

Fig.5. Western blot method using both antigens of type IV collagen (derived human placenta, pepsin treated) and NC1(derived bovine renal glomeruli, collagenase

treated):

anti NC1 mono 12D: anti NC1 monoclonal antibody;

anti NC1 poly: anti NC1 polyclonal antibody;

anti type IV: anti human type IV collagen polyclonal antibody;

control: no addition of the primary antibody

Table 1. Staining procedure

Table 1.

Materials;

Frozen renal tissues of crab-eating macaque anti GBM antibody nephritis model and normal one. (Embed into OCT compound, freeze rapidly using dry ice-acetone or liquid nitrogen, preserve at −80℃).

Antibody;

Primary antibody: Anti NC1 monoclonal antibody(derived from mouse);

Secondary antibody: FITC labeled anti mouse antibody(derived from rabbit) (DAKO Inc., Code No. F0232, Lot. 045).

Procedure;

1) Prepare frozen slice pieces using Cryostatt
2) After drying, fix them for 5 minutes with acetone
3) Wash with phosphoric acid buffer solution(PBS, pH 7.4)
4) Incubate for 2 hours at room temperature with the primary antibody (diluted into 500-fold solution)
5) Wash with PBS
6) Incubate for 1 hour at room temperature with the secondary antibody (diluted into 50-fold solution)
7) Wash with PBS
8) Mount with glycerol